

GUP1* and its close homologue *GUP2*, encoding multi-membrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisiae

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Summary

Many yeast species can utilise glycerol, both as sole carbon source and as an osmolyte. In *Saccharomyces cerevisiae*, physiological studies have previously shown the presence of an active uptake system driven by electrogenic proton symport. We have used transposon mutagenesis to isolate mutants affected in the transport of glycerol into the cell. Here we present the identification of *YGL084c*, encoding a multi-membrane-spanning protein, as being essential for proton symport of glycerol into *Saccharomyces cerevisiae*. The gene is named *GUP1* (Glycerol UPtake) and is important for growth on glycerol as carbon and energy source, as well as for osmotic protection by added glycerol, of a strain deficient in glycerol production. Another ORF, *YPL189w*, presenting a high degree of homology to *YGL084c*, similarly appears to be involved in active glycerol uptake in salt-containing glucose-based media in strains deficient in glycerol production. Analogously, this gene is named *GUP2*. To our knowledge, this is the first report on a gene product involved in active transport of glycerol in yeasts. Mutations with the same phenotypes occurred in two other open reading frames of previously unknown function, *YDL074c* and *YPL180w*.

Introduction

Osmotic stress is known to trigger a set of cellular responses which enable *Saccharomyces cerevisiae* to adapt to changes in the environment (Varela and Mager, 1996). When *S. cerevisiae* is exposed to osmotic stress, it responds by accumulating glycerol in the cytoplasm, up to molar concentrations, which counteracts cell dehydration. Glycerol is synthesised from dihydroxyacetone phosphate in two steps that branch off from glycolysis or gluconeogenesis. In the first step, dihydroxyacetone phosphate is converted into glycerol 3-phosphate in a reaction catalysed by a NAD-dependent glycerol 3-phosphate dehydrogenase. Two isoenzymes exist, encoded by *GDP1* and *GPD2* (Larsson *et al.*, 1993; Albertyn *et al.*, 1994; Eriksson *et al.*, 1995); however, only expression of *GPD1* is stimulated by osmotic stress. Similarly, two isoenzyme forms exist for the glycerol 3-phosphate-specific phosphatase, encoded by *GPP1* and *GPP2* (Norbeck *et al.*, 1996), which catalyses the final formation of glycerol. Also in this case only one of the genes, namely *GPP2*, is under osmotic regulation. Strains deleted for both *GPD1* and *GPD2* can no longer

synthesise glycerol, and they become highly sensitive to osmotic stress. However, Albertyn *et al.* (1994) observed that a *gpd1* strain upon salt stress is able to increase the intracellular glycerol level by taking up extracellular glycerol against a concentration gradient, indicating that the cell has an active uptake system for glycerol. Furthermore, R. Ansell and S. Hohmann (personal communication) found that the presence of small amounts of glycerol in the medium lowers the osmo-sensitivity of strains deficient in glycerol synthesis. This observation makes an important basis for screening for mutants deficient in active glycerol uptake, used in the present work.

S. cerevisiae is also able to utilise glycerol as sole source of carbon. The catabolic pathway involves a glycerol kinase encoded by *GUT1* (Sprague and Cronan, 1977; Rønnow, 1992; Pavlik *et al.*, 1993), and a mitochondrial glycerol 3-phosphate dehydrogenase encoded by *GUT2* (Sprague and Cronan, 1977; Rønnow and Kielland-Brandt, 1993). Mutants lacking one of these genes are not able to use glycerol as carbon source. Some yeast species, including *Schizosaccharomyces pombe*, have an alternative catabolic pathway (May *et al.*, 1982; Gancedo *et al.*, 1986), in which glycerol is first converted into dihydroxyacetone by a dehydrogenase and further to dihydroxyacetone phosphate by a kinase. Until recently, this pathway was thought not to be present in *S. cerevisiae*. However, results by Norbeck and Blomberg (1997) and Blomberg (1997) suggest that these enzymes might also exist in *S. cerevisiae*, although their function is unknown.

Physiological characterisation of glycerol uptake in *S. cerevisiae* has shown that glycerol enters the cell by at least two different mechanisms depending on the growth conditions. When glucose is present, glycerol can enter the cells by facilitated transport, probably through the glycerol channel, encoded by the *FPS1* gene (Van Aelst *et al.*, 1991; Luyten *et al.*, 1995; Sutherland *et al.*, 1997; Tamas *et al.*, 1999). However, in ethanol-, glycerol- and acetate-grown cells, a proton symport system ensures uptake of glycerol (Lages and Lucas, 1997; Sutherland *et al.*, 1997).

To understand the mechanisms behind glycerol uptake in *S. cerevisiae*, as well as to identify other proteins important for glycerol catabolism, we isolated mutants deficient in uptake and/or catabolism of glycerol, using a parental strain unable to produce glycerol. With a mTn-*lacZ*/*LEU2*-mutagenised library set-up (Ross-Macdonald *et al.*, 1995), mutants were isolated by two different screens. In the first screen, mutants that were no longer able to utilise glycerol as sole carbon source were selected. In the second screen we isolated mutants which lack the ability to survive salt stress even when glycerol is available in the medium. This screen was included in order to select mutants directly affected in genes encoding proteins important for the active uptake of glycerol. We isolated and identified mutants in four different loci, of which only one, *SPT7*, has previously been assigned

a function in the utilisation of glycerol as carbon source (Winston *et al.*, 1984). The three other ORF's all encode unknown putative proteins. One of these, *YGL084c*, has been suggested to encode a multi-membrane-spanning protein belonging to the major facilitator superfamily (Nelissen *et al.*, 1997). Our results show that *YGL084c* is essential for proper growth on glycerol, as well as for glycerol-mediated recovery from salt stress. A highly homologous ORF, *YPL189w*, turned out to be important for glycerol uptake in glucose-grown cells when exposed to salt stress. We propose that both genes encode proteins which have the ability to actively transport glycerol into the cell, *YGL084c*, in particular, being responsible for the previously described glycerol-proton symporter activity in *S. cerevisiae*.

Results

Isolation of mutants defective in uptake/catabolism of glycerol

To identify proteins involved in active uptake of glycerol, as well as to identify novel proteins necessary for the catabolism of glycerol, we subjected a *gpd1 gpd2* mutant (YSH6.142-3D), which lacks both isoenzymes of cytoplasmic glycerol 3-phosphate dehydrogenases, and is therefore unable to produce glycerol, to transposon mutagenesis. The parental strain was transformed with a mixture of yeast genomic fragments cut out from an mTn-*lacZ/LEU2*-mutagenised plasmid library (Ross-Macdonald *et al.*, 1995; see Experimental procedures). The efficiency of yeast to integrate linear DNA into homologous regions of the genome ensures that each integrant becomes a mutant having a transposon somewhere in the genome, quite often disrupting a coding region. Two different screens were used to select mutants of interest. In one, we picked mutants that were no longer able to grow with 2% glycerol as sole carbon and energy source (*gut*-diagnostic medium). In the second screen, we isolated mutants lacking glycerol-mediated recovery from salt stress on rich medium with 1 M NaCl, containing glucose as carbon source and supplemented with 10 mM glycerol. After transformation in the *gpd1 gpd2* background with the inserts of the Tn-library, around 10,000 colonies were isolated on synthetic complete medium without leucine, selecting for integration of the transposon. All colonies were subsequently screened for both phenotypes by replica plating to both *gut*-diagnostic plates, containing glycerol as sole carbon source, and to YPD plates containing 1 M NaCl and 10 mM glycerol. After several repetitive rounds of screening in both set-ups and

elimination of mutants with a general growth phenotype, we ended up with 5 mutants (Table 1), satisfying the criteria of both screens, i.e. all five mutants both lack glycerol-mediated recovery from salt stress and have reduced ability to utilise glycerol as carbon source (*gut*) (Fig. 1). None of the mutants were petites as they all grow with lactate and ethanol as carbon source (data not shown). Using a so-called rescue plasmid set-up (Ross-Macdonald *et al.*, 1995), we recovered transposon sequences together with sequences from the flanking regions, thereby identifying four genes that were disrupted by the transposon: *SPT7*, *YDL074c*, *YPL180w* and *YGL084c* (*GUPI*) (Table 1). Only *SPT7* has earlier been assigned a function (Winston *et al.*, 1987).

In transposon mutant BHY23, the transposon had been inserted approximately in the middle of *SPT7*. The gene encodes a 1332 amino acid residue nuclear protein. *SPT7* is one of the *SPT* genes, originally identified by mutations acting as suppressors of certain *Ty* insertions which affect transcription start site selection (Winston *et al.*, 1984; Gansheroff *et al.*, 1995). *spt7* mutants are known to grow slowly on several carbon sources, including glycerol.

The three other Tn-tagged ORF's had not been assigned any function yet. *YDL074c* encodes a 700 amino acid residue putative protein. It was recovered twice, through BHY13 and BHY24, with the transposon inserted at the same position, namely after nucleotide 1463. Curiously, the two mutants show slightly different phenotype, suggesting a second mutation in one of them. Search in the databases suggests some homology between Ydl074cp and proteins engaged in interaction with microtubules. In BHY21, the transposon is located in the very C-terminal end of ORF *YPL180w*, which encodes a 799 amino acid residue putative protein. *YPL180w* shows no significant homology to any protein in the databases.

In mutant BHY22, the transposon is located in the N-terminal end of an open reading frame, *YGL084c*, encoding a 560 amino acid residue putative protein. *YGL084c* has recently been suggested to encode a multi-membrane-spanning protein of unknown function, belonging to the major facilitator superfamily (Nelissen *et al.*, 1997). The predicted transmembrane nature of Ygl084cp and the observed phenotypes of BHY22 make Ygl084cp an obvious candidate for an active glycerol transporter, or perhaps a sensor. The reason for this lies in the fact that the mutant affected in this gene is deficient in both glycerol-mediated recovery from salt stress and in growth on glycerol, indicating a role in uptake. Therefore we called the gene *GUPI*, for Glycerol Uptake. The name has been reserved in the Saccharomyces Genome Database in Stanford. Blast searches in the databases revealed two proteins with significant homology to the predicted amino acid sequence of Ygl084cp, namely Ypl189wp, a 608 amino acid residue putative protein also from *S. cerevisiae*,

and the one encoded by *SPAC24H6.01c*, an ORF of 231 codons from *Schiz. pombe*. At the amino acid level the sequence identity between *YGL084c* and *YPL189w* is 57% and the similarity is 77%, suggesting closely related functions (Fig. 2, panel A). The somewhat smaller putative ORF from *Schiz. pombe* is homologous (49% identity) to the N-terminal part of *YGL084c* (Fig. 2, panel A). All three proteins are predicted to be membrane proteins, Ygl084cp and Ypl189wp having 8-10 putative membrane-spanning domains, and Spac24H6.01cp only four. By comparing the hydrophobicity plots (Kyte and Doolittle, 1982) of these proteins, the similarity is also quite obvious (Fig. 2, panel B).

Phenotypes of gup1

To analyse further the phenotype of *gup1*, we cloned a chromosomal copy of *GUP1* with 1421 nucleotides of the promoter region, using homologous recombination/GAP-repair (Orr-Weaver and Szostak, 1983; Rothstein, 1991; see Experimental procedures). By using this strategy we ensured cloning with the high fidelity characteristic of *in vivo* replication. As can be seen in Fig. 3 (panel A), introduction of a low-copy (centromere-based) plasmid (pBH2178) containing *GUP1* into strain BHY29-2C complements both the *gut* (glycerol utilisation) phenotype and the ability to recover from salt stress when glycerol is present in the medium. Over-expression of *GUP1* from a high-copy (2 μ -based) plasmid does not lead to enhanced growth on *gut*-diagnostic plates (glycerol as carbon and energy source), or on YPD containing 1 M NaCl and supplemented with 10 mM glycerol. However, the zone in which the cells can grow on YPD + 1 M NaCl, around a filter disk containing glycerol, is clearly enlarged when a high-copy plasmid with *GUP1* has been introduced into BHY29-2C cells, as compared to the low-copy situation (Fig. 3, panel B). To see if this ability also results in a general higher osmo-tolerance, growth experiments on plates with rich medium with increasing salt concentration were performed. However, there was no improved osmo-tolerance when *GUP1* was overexpressed, relative to expression from a low-copy plasmid (data not shown).

To analyse the effect of a *gup1* deletion in an otherwise wild-type background, we next made a one-step deletion/disruption of *GUP1* by insertion of the *his5*⁺ gene from *Schiz. pombe*, as described in Experimental procedures. The phenotypes of the resulting strain, BHY54, on different media were analysed by a drop test, where dilution series of cells are spotted on plates (Fig. 4).

Whereas there was no effect when lactate/ethanol were tested (data not shown), deletion of *GUP1* gave a clear phenotype on glycerol (Fig. 4, upper panel), implying the importance of Gup1p in the utilisation of glycerol as carbon source, and not a common defect in utilising non-fermentable carbon sources. Unexpectedly, we also saw a phenotype when the *gup1* mutant was growing with glucose as carbon source in the presence of osmotic stress (Fig. 4, lower panel). This phenotype was more pronounced when the cells were exposed to osmotic stress with 1 M NaCl than with 1 M KCl (data not shown). Addition of 50 mM glycerol to the 1 M NaCl plates did not remedy the phenotype. Sorbitol, 1.5 M, leading to about the same decrease in water activity, had almost no effect on growth (data not shown). Introduction of a low-copy plasmid with *GUP1* under control of its own promoter into a *gup1* strain restores wild-type growth in all cases (Fig. 4 and data not shown). These observations suggest that even in a wild-type background, i.e. with no impairment in glycerol synthesis, Gup1p, and thus probably also some (re)uptake of glycerol, is necessary for proper growth under osmotic stress. However, it should be kept in mind that the effect of lacking Gup1p is much more dramatic when glycerol synthesis is blocked (Fig. 3A, lower panel, lane 2). Introduction of a multi-copy plasmid with *GUP1* into BHY54 did not give any enhanced growth, as compared to a single-copy plasmid with *GUP1*, on any of the media tested (Fig. 4 and data not shown).

Deletion of YPL189w

As previously mentioned, screening of the Saccharomyces Genome Database revealed Ypl189wp to be a close homologue of Gup1p. The high degree of identity, 57% at the amino acid level, suggests that the homologue might serve a very similar function in the cell. To study the function of Ypl189wp, we made deletion mutant strains in which the coding region, except for the first 10 amino acid residues, was replaced by the heterologous marker for geneticin resistance, *kanMX* (Wach *et al.*, 1994). The deletion was made in both W303-1A and BHY54, which already lacks *GUP1*. The phenotypes of the deletion mutants (CLy5 and CLy3, respectively) were tested by drop assays on several different carbon sources. Deletion of *YPL189w* did not cause any impairment of growth when tested in glucose, glycerol or ethanol, as compared with the corresponding parental strains (data not shown). Also when exposed to osmotic stress on glucose-based media, by the addition of 1M NaCl, 1M KCl or 1.5 M sorbitol, no effects were seen. In liquid media with glucose,

ethanol or glycerol as sole carbon source, deletion of *YPL189w* did not cause significant changes in the specific growth rate (data not shown).

We also tested the ability of *YPL189w* to complement a deletion of *GUP1*. Using PCR with chromosomal DNA as template, followed by homologous recombination/GAP-repair, we cloned *YPL189w* with its 789-bp promoter region and inserted it into both a centromere-based vector and a 2 μ -based vector. However, neither on *gut*-diagnostic plates nor on YPD plates containing 1 M NaCl was overexpression of *YPL189w* able to complement the deletion phenotypes of the *gup1* mutant (data not shown).

It should be noted that these experiments were carried out with a strain with functional glycerol synthesis. Later experiments, to be described below, revealed a phenotype for *yp1189w* in a strain defective for *gpd1*, *gpd2* and *gup1*.

Involvement of Gup1p in active transport of glycerol in ethanol-grown cells

Earlier studies on the transport of glycerol in ethanol-grown cells showed that active glycerol uptake in *S. cerevisiae* is accompanied by a simultaneous uptake of protons (Lages and Lucas, 1997; Sutherland *et al.*, 1997). Both studies showed that active glycerol uptake was only seen in glucose-derepressed cells. In glucose-grown cells, on the other hand, glycerol enters the cell through the constitutively expressed Fps1p, which has been suggested to mediate glycerol uptake by facilitated diffusion (Sutherland *et al.*, 1997), and which responds to salt stress by closing, thereby causing glycerol retention (Tamás *et al.*, 1999). Under both repressing and derepressing conditions, simple diffusion through the membrane with a similar passive diffusion coefficient was also measured. The phenotypes of the *gup1* mutant isolated here suggested that Gup1p could be responsible for the active uptake of glycerol. Therefore, we analysed the uptake of glycerol in both the parental strain W303-1A and the *gup1* mutant after growth on ethanol-based complex medium. Remarkably, Eadie-Hofstee plots of the glycerol uptake in both the wild-type and the *gup1* mutant showed that a saturable uptake system was still apparent after deletion of *GUP1*, with an approximately similar K_m and with the V_{max} only decreased around 35% (Fig. 5; Table 2). No further significant changes were seen when the *gup1* mutation was combined with either a deletion of the gene for the glycerol channel, *FPS1*, or a deletion of the gene for the close homologue of Gup1p, *YPL189w*. However, the glycerol-induced proton signal was not detectable in any of the

three *gup1* mutant strains (data not shown). Furthermore, the addition of the protonophore CCCP did not prevent the residual saturable glycerol uptake. Although the V_{\max} for glycerol uptake in the *gup1* mutant strain grown on ethanol is still around 65% of the wild-type level, the defect in uptake is obviously sufficient to affect the utilisation of glycerol as carbon and energy source. One explanation for the residual glycerol uptake in the *gup1* mutant could be that there exists an additional uptake system with Michaelis-Menten kinetics. Another explanation could be that the residual uptake is not active as such, but is pulled by an active catabolism of glycerol under the derepressing conditions. Glycerol destined for utilisation is initially phosphorylated by a glycerol kinase, encoded by *GUT1* (Sprague and Cronan, 1977; Pavlik *et al.*, 1993). Consistently, the levels of kinase activity measured in extracts from either W303-1A or *gpd1Δ gpd2Δ* strains, as well as in extracts from *gup1Δ* and *gup1Δ ypl189wΔ* strains were high, 66.1 ± 4.1 and 55.8 ± 11.4 mU mg protein⁻¹ for the first two strains and a somewhat lower activity of 43.1 ± 5.8 and 48.9 ± 13.5 mU mg protein⁻¹ for the last two strains, respectively. In order to clarify whether glycerol kinase has an effect on the apparent kinetics of uptake, we deleted *GUT1* both in a wild type and a *gpd1* mutant background (Fig 5; Table 2). In both these strains lacking *GUT1*, V_{\max} for glycerol uptake is decreased relative to the wild type level (26 % and 11 %, respectively), indicating that the kinase to some extent affects the uptake kinetics. We also deleted *GUP1* in different combinations with strains already lacking *GUT1* and compared kinetics for the glycerol uptake under derepressing conditions (Fig. 5; Table 2). Interestingly, in neither the *gut1 gpd1 gup1* nor the *gut1 ypl189w gup1* strains, any saturable uptake can be measured, leaving only simple diffusion. This indicates that the glycerol uptake measured in W303-1A, besides being a mixture of active uptake and passive diffusion, also is kinetically influenced by the first step in catabolism.

To investigate if other proteins which are known to be involved in glycerol metabolism are important for uptake of glycerol under derepressing conditions, we characterised uptake in strains lacking these enzymes (Table 3). The only strains with a measurable decrease in V_{\max} appear to be the *gut1* strain and the *gut2* strain. Thus, the saturability of glycerol uptake seen in *gup1* mutants, as seen in Table 2 and Fig. 5, appears to be a consequence of the catabolism of glycerol only.

One may wonder how the relatively modest decrease in uptake in a *gup1* mutant allowed us to pick it up in the glycerol utilisation screen. We have not done much to answer this question, but it may be noted that the media are not identical; the plates are mainly defined, containing only 0.05% peptone, while the growth media for uptake assays are complex.

We also analysed the roles of Gut1p (glycerol kinase) and Gup1p in the ability to

accumulate radioactivity provided as [^{14}C]glycerol against a concentration gradient, after growing the cells under derepressing conditions (Fig. 6). Deletion of *GUP1* in the W303-1A genetic background clearly reduces the in/out ratio along incubation time, without reaching a *plateau*, supporting the notion that this strain has a reduced ability to actively take up glycerol. Furthermore, in this case the addition of the protonophore CCCP did not cause loss of radioactivity, consistently with the interpretation that glycerol kinase has converted the radioactivity from the form of glycerol to other chemical forms. This drug also did not allow incorporation of radioactivity into the cells, consistently with its long-term general inhibitory effect on metabolism, affecting the ATP level and intracellular enzymes, in particular glycerol kinase. On the other hand, the effect of deletion of *GUT1* allows the establishment of the distribution of free radiolabelled glycerol in accordance with active uptake alone, i.e., without the involvement of metabolism. In this case, addition of CCCP causes a rapid relaxation of the in/out ratio to unity, as expected for an active transport system using the proton gradient.

Involvement of Gup1p and its close homologue in active transport in glucose-grown cells

As mentioned above, all the selected mutants, besides being defective in growth on glycerol, were also deficient in glycerol-mediated recovery from salt stress in glucose medium. To establish this further, we investigated glycerol uptake in cells grown on glucose supplemented with 1 M NaCl and 15 mM glycerol, as well as in cells grown on glucose alone as a control (Table 4). All strains presented in Table 4 resumed growth after widely different lag phases upon exposure to the glucose-salt-glycerol medium. Recovery from the lag was reproducible and found not to be due to selection of spontaneous mutants, but we did not investigate whether glycerol synthesis via dihydroxyacetone could be an explanation. In accordance with earlier studies (Lages and Lucas, 1997; Sutherland *et al.*, 1997), no glycerol transport was detected in cells growing in non-stressing, glucose-based medium. The same absence was verified in cells grown under osmotic stress, but harbouring a functional glycerol synthesis pathway. Importantly, however, cells deleted in *GPD1* or both *GPD1* and *GPD2*, presented glycerol uptake when growing on glucose in the presence of salt stress (Table 4). Kinetic parameters measured in this last strain under these conditions revealed the same K_m but a higher V_{max} (3 to 4 times) than in derepressed wild-type cells (compare Tables 4 and 2). The original transposon-tagged mutant, BHY22 (*gpd1 Δ gpd2 Δ gup1 Δ*) grows very poorly on

glucose supplemented with 1 M NaCl and 10 mM glycerol (Fig. 1). In this strain, V_{\max} was 60% of the value measured in *gpd1 gpd2* alone (Table 4). Interestingly, when we combined the *gpd1* and *gup1* mutations with a deletion of the *GUP1* homologue, *YPL189w*, no transport is measured (Table 4), indicating that this gene is responsible for the residual uptake, present after deleting *GUP1*, under these growth conditions. The Ypl189wp-mediated transport system presents a K_m very similar to that of Gup1p, 1.3 mM.

Because glycerol kinase has an important role in the apparent kinetics of glycerol uptake into cells grown on ethanol, we considered the possibility that it might also have a role in the glucose-grown, salt-stressed cells. Although this was, *a priori*, unlikely since the glycerol kinase level is low in glucose-grown cells (Sprague and Cronan, 1977), we nevertheless measured glycerol kinase activity in all strains listed in Table 4 grown on glucose and on glucose with salt and glycerol. No salt induction was seen, and all values were low, less than 10% of that seen in the wild type grown on ethanol (data not shown). We conclude that glycerol kinase is unimportant for glycerol uptake in glucose-grown, salt-stressed cells.

A glycerol transport system of the proton symport type should, as described earlier (Lages and Lucas, 1997), display proton uptake upon glycerol addition. Strains presenting glycerol uptake when exposed to salt stress also displayed significant glycerol-dependent proton uptake, while the wild type (W303-1A) did not, indicating that both Gup1p and Ypl189wp can correspond to such type of active transport system. According to (1) the molecular homology between these two genes, and (2) the apparent similarity of behaviour, as far as transport is concerned, we propose the *YPL189w* gene to be named *GUP2*.

The experiment presented in Table 4 shows that *GUP1*-dependent and *GUP2*-dependent glycerol uptake in glucose-grown cells is tightly controlled and requires the extreme conditions of the rather strong osmotic stress in 1 M NaCl combined with a deficiency in glycerol synthesis. When the *GPD2* gene was deleted in a strain already partially deficient in glycerol synthesis because of a *gpd1* deletion, the *GUP2*-dependent glycerol uptake went up by a factor of two (Table 4), indicating an extension of the same kind of control. On the other hand, the differences between *gup1* and *gup2* phenotypes suggest the two genes to be differently regulated. In this connection it may be noted that we found that Gup1p is necessary to shorten lag phase in salt-stressed cells growing on glucose: A *gup1* mutant has a lag phase 2.5 times longer than the corresponding *GUP1* strain, whether the latter be *GPD1 GPD2* (W303-1A) or *gpd1 gpd2* (data not shown). Whether the appearance of glycerol uptake in the salt-stressed cells caused by defective glycerol synthesis is due

to an enhancement of the already high osmotic stress, or rather a direct controlling effect of low intracellular glycerol concentration, or perhaps more indirect mechanisms, remains to be investigated.

Localisation of Gup1p

A gene fusion encoding a hemagglutinin-tagged version of Gup1p was constructed in order to determine cellular location(s) of the protein. The codons for the hemagglutinin (HA)-tag were inserted into an introduced restriction site five codons upstream of the stop codon, and the construct was verified by sequencing. The construct was made so that Gup1p-HA is under the control of its own promoter on a centromere-based plasmid. The construct was able to complement the growth defect of *gup1* on glycerol-based media (data not shown), indicating that at least enough of the tagged protein had the location needed for function. On a Western blot, Gup1p-HA appears as a double band at ~ 45 kDa. This is lower than expected from the sequence (65.3 kDa; Saccharomyces Genome Database, Stanford). A higher electrophoretic mobility for hydrophobic membrane proteins is, however, not uncommon since they bind excessive amounts of SDS (Pinson *et al.*, 1996).

HA-Gup1p was next expressed in a strain (W3124) in which the genes encoding the three major vacuolar proteases; proteinase A, proteinase B and carboxypeptidase Y have been deleted to minimise unwanted proteolysis. The co-localisation of the tagged Gup1p with known marker enzymes for cellular organelles in a sucrose gradient was subsequently investigated. The cells were grown to mid-exponential phase, broken with glass beads and the membrane fraction loaded onto a continuous sucrose gradient (20% - 55%). Thirty fractions were collected and the enzymatic activities of known marker enzymes determined (Fig. 7). The plasma membrane was detected by measuring H⁺-ATPase activity, the endoplasmic reticulum by assay of NADPH-cytochrome c reductase, mitochondria by monitoring cytochrome c oxidase activity and vacuolar membranes by measuring α -mannosidase (Villalba *et al.*, 1992). The distribution of Gup1p was determined by Western blotting and subsequent quantification of the bands (ImageMaster, Amersham Pharmacia). Gup1p peaks between 40 and 45%, which corresponds to the second major peak of the plasma membrane. A minor Gup1p peak is also present at ~ 38%; this could correspond to the first plasma membrane peak; however, it might also represent an endoplasmic reticulum (ER) or mitochondrial

localization (Fig. 7).

Discussion

Saccharomyces cerevisiae has earlier been found to possess the ability to take up glycerol from the surroundings against a concentration gradient (Lages and Lucas, 1997). The uptake was shown to be driven by electrogenic proton symport. Here we describe the identification and characterisation of an open reading frame, *YGL084c*, which we propose to encode a protein, Gup1p, involved in active uptake of glycerol. A homologous gene, *YPL189w*, *GUP2*, was found to have the same function under extreme conditions.

Using a mTn-*lacZ*/*LEU2*-mutagenised library (Ross-Macdonald *et al.*, 1995), we isolated mutants defective in either utilisation of glycerol as carbon source and/or in glycerol-mediated recovery from salt stress as determined in the absence of glycerol production. Of the four different loci found to be hit in the mutants satisfying both screens, only *SPT7* has earlier been assigned a function. *SPT7* belongs to a group of genes known to be affected in transcription start site selection (Gansheroff *et al.*, 1995). One of the phenotypes of these mutants is slower growth on several carbon sources, including glycerol. The three other ORF's all encode putative proteins with unknown function. One of the ORF's, *YGL084c*, has been suggested to encode a multi-membrane-spanning protein belonging to the major facilitator superfamily (Nelissen *et al.*, 1997). We call the gene *GUP1*, for Glycerol Uptake. From the deduced amino acid sequence, 8-10 transmembrane domains can be predicted, very similarly to the close homologue of *GUP1*, *YPL189w*.

The predicted transmembrane nature of Gup1p and Ypl189wp, together with the phenotypes of the isolated transposon mutants, made these ORF's obvious candidates for further studies, in particular the one from *GUP1*. The ability of *GUP1* to complement the phenotypes of the transposon mutant (Fig. 3) was verified by cloning of a wild-type copy of *GUP1* by GAP-repair. In an otherwise wild-type background, deletion of *GUP1* results in slow growth on glycerol as sole carbon source. However, the *gup1* mutant also has a moderate growth defect on rich media with glucose as carbon source when supplemented with 1 M NaCl, which may be interpreted to mean that even when glycerol production is not affected, salt stress requires reuptake of glycerol, leaking out of the cells. On the other hand, active uptake of glycerol under these conditions was only detected in strains with a full or partial block in glycerol synthesis.

An integral membrane localization of Gup1p is consistent with a direct role in the active uptake of glycerol. A further prediction of this role would be localisation of at least some of the molecules to the plasma membrane. We therefore separated membranes on a sucrose gradient. HA-

tagged Gup1p was found in two peaks that coincided with the fractions containing plasma membrane. We considered the possibility that the minor one of the two Gup1p peaks could represent either the ER membrane or the mitochondrial membrane. However, as both these organelles also are present at other sucrose concentrations with no corresponding peak in the Gup1p profile, we find these localisations unlikely. We interpret the fact that the Gup1p profile does not coincide completely with the plasma membrane, in the beginning and the end of its curve, to be due to difficulties in quantification of the Western blotting.

The high degree of similarity between Gup1p and its close homologue (~57% identity and ~77% similarity), encoded by *YPL189w*, suggested that the latter might fulfil a very similar function. However, when colony growth was assayed, neither deletion nor high-copy expression of *YPL189w* had any effect. Further, no complementation was observed in experiments where *YPL189w* was expressed from a high-copy vector in a strain lacking *GUP1*. Over-expression with a strong promoter was not investigated. β -galactosidase assays of promoter fusions to *lacZ* indicate that both promoters are weak, with the *YPL189w* promoter being around ten fold weaker than the *GUP1* promoter (data not shown). Importantly, however, *YPL189w* was necessary for a residual active glycerol uptake in salt-stressed *gpd1 gup1* cells on glucose. We therefore named this gene *GUP2* as mentioned above.

Previous kinetic studies of cells not repressed by glucose have shown glycerol uptake to fit the sum of one diffusion component and one active saturable system (Lages and Lucas, 1997; Sutherland *et al.*, 1997). Our uptake kinetics for the wild type is very similar to these data (Table 2). However, in the *gup1* mutant, which is severely impaired in its growth with glycerol as carbon source, we still found kinetics of glycerol uptake with both a diffusion component and a saturable component, although we observed changes in V_{\max} . It is noteworthy, however, that electrogenic proton symport could no longer be measured in any of the *gup1* mutants, and that the addition of the protonophore CCCP did not totally prevent the residual uptake. We reasoned that the residual, *GUP1*-independent, uptake was probably not active as such but pulled by the metabolic trapping of incoming glycerol by glycerol kinase, encoded by *GUT1*, influencing the apparent uptake kinetics. This was analysed further by comparing various combinations of genes including *gut1* and *gup1* as well as controls. Deletion of *gut1* either alone or in combination with *gpd1*, only leads to smaller changes in V_{\max} . However, when the effect of *gup1* was studied in a *gut1 gpd1* background, there was a dramatic change in the kinetics of glycerol uptake (Table 2; Fig. 5). In this background, defective in glycerol synthesis and glycerol utilisation, the diffusion constant was unaffected,

whereas the uptake with saturable kinetics was eliminated by deletion of *GUPI*.

Another way to analyse the components of the uptake of glycerol is to measure glycerol accumulation against a concentration gradient. The in/out accumulation ratios in Fig. 6 show that the overall uptake of glycerol consists of both active uptake by Gup1p and metabolic trapping through the action of the kinase, Gut1p. Probably because of the time course of these experiments, the catabolism has a major influence. Thus, the major part of the accumulation over time corresponds to conversion of glycerol in metabolism, which fits with the lack of efflux when CCCP is added. Moreover, in a *gut1* strain background, Gup1p was responsible for most of the overall rate of accumulation. Consistently, all of the accumulated glycerol diffused out upon addition of CCCP.

Both the uptake and accumulation results show that under derepressing conditions the glycerol uptake is the sum of a saturable, active uptake, and diffusion, and that the latter is strongly influenced by glycerol kinase, giving rise to an apparently saturable component in *gup1* strains. A sufficiently short uptake experiment might, in principle, overcome the kinetic artefact caused by glycerol kinase. However, in contrast to the situation with glucose, the uptake of which can be measured during less than 0.2 seconds (Walsh *et al.*, 1994), rates of glycerol uptake are insufficient for such short uptake experiments. It is also conceivable that uptake kinetics is complicated by direct interactions between proteins involved in glycerol transport and glycerol metabolism, like the situation in *Escherichia coli* (Voegelé *et al.*, 1993), but we have no strong evidence for such interactions. Nevertheless, measurements of the kinase activity in ethanol-growing cells indicate that full kinase activity might be connected with functional Gup1p. We can thus not exclude the possibility that there is some kind of direct interaction between Gup1p and Gut1p, affecting the activity of one or both. Despite the importance of the glycerol kinase when accumulation of radioactivity from glycerol is measured, it is clear that diffusion coupled to the metabolism is not sufficient for the cells to grow properly on glycerol as carbon and energy source, implying the physiological importance of active uptake through Gup1p.

Thus, here we describe the identification of the gene *GUPI*, which is essential for proper growth on glycerol, as well as for glycerol-mediated recovery from salt stress. *GUPI* probably encodes a multi-membrane-spanning protein belonging to the major facilitator superfamily (Nelissen *et al.*, 1997). By separating membranes on a sucrose gradient, we have shown that Gup1p is very likely to be present at least partly in the plasma membrane fraction.

As has earlier been seen for wild type cells (Lages and Lucas, 1997; Sutherland *et al.*, 1997), we did not see any active glycerol uptake in any of the investigated strains when the cells

were grown on glucose. However, when we analysed cells growing on glucose supplemented with 1 M NaCl and 15 mM glycerol, strains that are deficient in glycerol synthesis (*gpd1* or *gpd1 gpd2* mutants), have a strong capacity to actively transport glycerol into the cells. Although the *gup1* mutation, in this genetic background, has a clear growth phenotype, it still allows some active uptake of glycerol, due to the function of *GUP2*.

Considering the following: (1) in ethanol cells only Gup1p is active, (2) *GUP1* is essential for growth on glycerol as sole carbon and energy source, (3) a *gup1* deletion increases lag phase duration of salt-stressed cells on glucose, (4) Gup2p activity is only apparent in salt-stressed cells on glucose, and (5) a *gup2* deletion appears not to affect growth any carbon source, either glucose or non-fermentable ones, these two genes must be differently regulated, at least to some extent. It is noteworthy that there are two differentially controlled isoenzymes of the first step of glycerol production (Eriksson *et al.*, 1995; Ansell *et al.*, 1997), two differentially controlled isoenzymes of the second step of glycerol production (Norbeck *et al.*, 1996) and now also two putative transporters of glycerol that are controlled in different ways, yet to be characterised.

In conclusion, we have found that under different metabolic conditions Gup1p and/or Gup2p are necessary for active uptake of glycerol, taking place by proton symport. We can not exclude the possibility that Gup1p and Gup2p have sensing or other regulatory functions, but the most straightforward hypothesis is that they are transporters of glycerol. This is supported by the fact that they belong to the major facilitator superfamily and by our subcellular fractionation being consistent with a plasma membrane localization.

Experimental procedures

Strains and media

The *S. cerevisiae* strains used in this study are listed in Table 5. BHY13, 21, 22, 23 and 24 were isolated as described below by transposon-based mutagenesis. BHY20-8C, 26-5D, 27-3B, 28-8C and 29-2C were isolated as spores after crossing of BHY13, 21, 22, 23 and 24, respectively, with UTL-7A. Strains with a deletion of either the *GUT1* or the *GUT2* gene were constructed by the two-step loop-in/loop-out technique (Rothstein, 1991). For constructing the *gut1* deletion, plasmid pCL370 was digested with *MluI* before introduction into W303-1A. CLy1 and CLy2, two

independent isolates from the same loop-in, are isogenic except for *gut1* in CLy1 and *GUT1* in CLy2. Similarly, for the *gut2* deletion, pBH2038 was digested with *HpaI* before introduction into W303-1A. BHY39 and BHY40, are two independent isolates from the same loop-in, being isogenic except for *gut2* in BHY40 and *GUT2* in BHY39. BHY51 was isolated after introduction and homologous recombination in YSH481 of a PCR-generated fragment containing the gene for geneticin resistance flanked by *GUT1* promoter sequences and *GUT1* downstream sequences (Wach et al., 1994; Wach, 1996). The desired disruption of the *GUT1* gene was verified by PCR. Strains lacking *YGL084c* (*GUP1*) were constructed as follows: After integrative transformation of W303-1A, YSH481, W303-1B and BHY51 with a *BsaI-XhoI* fragment from plasmid pBH2340, containing the *his5⁺* gene from *Schizosaccharomyces pombe* flanked by *YGL084c*-flanking sequences, BHY54, BHY56, BHY58 and BHY66 were selected on SC plates lacking histidine. The desired deletion was verified by PCR. BHY52 was isolated after integration in strain W303-1A of a *Sall-XbaI* fragment from plasmid *fps1Δ::HIS3*-long (obtained from M. Tamás), in which 2.3 kb of the coding region of *FPS1* has been replaced by the *HIS3* gene. To isolate a *fps1Δ gup1Δ* double mutant, BHY52 and BHY58 were crossed and tetrads were dissected. Using PCR, BHY61-1A was found to lack both genes. Similarly, a *gut1Δ gup1Δ* double mutant was made by crossing BHY61-1A and CLy1, and PCR showed BHY65-1C to lack both genes. To construct the *gut1Δ gup1Δ ypl189wΔ* strain, BHY67, the *gpd1Δ gup1Δ ypl189wΔ* strain, CLy4, the *gup1Δ ypl189wΔ* strain, CLy3 and the *ypl189wΔ* mutant, CLy5, *YPL189w* was deleted by transformation and selection for recombinants on geneticin, using a *BstBI-BsrBI* fragment from pCL448 in strains BHY65-1C, BHY56, BHY54 and W303-1A, respectively. Desired substitution was confirmed by PCR.

Batch cultures of yeast were grown aerobically at 30°C in standard complex or defined media (Rose *et al.*, 1990). For sporulation, pre-sporulation medium containing 0.8% yeast extract, 0.3% peptone and 10% glucose, and sporulation medium, 1% potassium acetate, 0.1% yeast extract and 0.05% glucose, were used. The inability to utilise glycerol as carbon and energy source (*gut* phenotype) was recognised on glycerol-based defined media supplemented with 0.05% peptone (*gut*-diagnostic medium, modified from Rønnow and Kielland-Brandt, 1993). For transport assays, cells were cultivated in yeast extract-peptone-based media (Rose *et al.*, 1990) with 2% (w/v) of ethanol or glucose as carbon sources.

Growth experiments on different plates were made as follows: Cells were inoculated in liquid medium with glucose carbon source. After dilution to OD₆₀₀ ~1, different dilutions were spotted on plates as indicated. The ability to grow was read after inoculation in 30°C for different

times as indicated. Osmo-tolerance were investigated as described above using plates containing increasing concentration of NaCl.

E. coli DH5 α (Sambrook *et al.*, 1989) was used for plasmid selection and propagation and grown on LB medium according to Sambrook *et al.* (1989). When needed for plasmid selection and maintenance, ampicillin or kanamycin was present at 100 μ g/ml.

Transposon-based mutagenesis

Library propagation, transformation and isolation of recombinants were essentially carried out as described by Ross-Macdonald *et al.* (1995) and http://ycmi.med.yale.edu/YGAC/insertion_libraries.html#mTn-lacZ/LEU2_library. From screening of around 10,000 colonies, 5 mutants were recognised by their inability to grow when replica plated onto glycerol-based (*gut*-diagnostic) medium and, in parallel, their inability to be rescued by 10 mM glycerol when exposed to 1 M NaCl on YP-based medium with 2% glucose as carbon source. All 5 mutants turned out to satisfy both criteria. Putative mutants were re-screened. To avoid mutants with generally impaired growth, and mutants defective in respiration, all mutants were also analysed for proper growth on YP-based medium with 2% ethanol as carbon and energy source. In order to identify the genes hit by the transposon, we first had to free the isolated mutants from the *URA3* gene. They were therefore crossed to yeast strain UTL-7A, and tetrads were dissected. Spores with the original mutant phenotypes and the *LEU2* marker but without the *URA3* marker were isolated and used as the source for rescue of the sequence flanking the transposon according to Ross-Macdonald *et al.* (1995).

Molecular cloning and plasmid construction

A chromosomal copy of *YGL084c* was cloned by a combination of PCR and homologous recombination/GAP-repair: A 1.6 kb fragment, covering from position -1560 to +38, relative to the start codeine, was amplified by PCR, introducing a *Bam*HI site in the downstream end. The fragment was blunt-end inserted into the *Eco*RV site in pBC sk+ (Stratagene), yielding pBH2100. Similarly, a 824 bp fragment, covering from position +1792 to +2515, relative to the start codeine,

was amplified and blunt-end inserted in the *EcoRV* site in pBC sk+ (Stratagene), yielding pBH2132. Recloning of an *NsiI*-*Bam*HI fragment from pBH2100 between the *Pst*I and *Bam*HI sites of pRS314 (Sikorski and Hieter, 1989) created pBH2128. After cutting pBH2132 with *Bst*EII, filling in the 5'-overhang and digestion with *Bam*HI, the resulting ~285 bp fragment was inserted into pRS316 (Sikorski and Hieter, 1989), which had previously been digested with *Xba*I and subjected to filling in of the 5'-overhangs and cleavage with *Bam*HI. This resulted in pBH2138. pBH2166 was constructed by recloning of a *Kpn*I-*Bam*HI fragment from pBH2128 between the same sites in pBH2138. pBH2178, containing the gap-repaired version of *YGL084c* along with its promoter, was made as follows: pBH2166 was digested with *Bam*HI and introduced into yeast strain W303-1A, selecting for *URA3*. After isolation from yeast cells, the plasmid was propagated in *E. coli*. Recloning of a *Kpn*I-*Sac*I fragment from pBH2178 between the corresponding sites in pRS306 (Sikorski and Hieter, 1989) and pRS426 (Christianson *et al.*, 1992) resulted in pBH2188 and pBH2198, respectively. pBH2200 was constructed after digestion of pBH2188 with *Hpa*I and *Spe*I, the 5'overhang was filled in and the linearised plasmid was subsequently re-ligated. pBH2209 was constructed by insertion of a 1 kb *Bam*HI fragment from pBH2100 into the *Bam*HI site in plasmid pFN8 (Nagawa and Fink, 1985). The entire promoter region of *YGL084c*, fused to *lacZ* in pBH2209 was sequenced and found to be identical to the already published sequence (Saccharomyces Genome Database, Stanford). pBH2340 was constructed as follows: A *Bgl*II-*EcoRV* fragment from pFA6a-GFPMT-HIS3MX6 (Wach *et al.*, 1997), containing the *his5*⁺ gene from *S. pombe*, was inserted into *Bgl*II- and *Hpa*I-digested pBH2188, replacing the whole *YGL084c* coding region with non-*Saccharomyces* DNA. A 574 bp fragment of sequences downstream of *YGL084c* was amplified by PCR, introducing an *Nco*I site just downstream of *YGL084c*. The obtained fragment was inserted into *Sma*I-digested pRS306 (Sikorski and Hieter, 1989), to give pBH2310. *YGL084c* was inserted to be under the control of the *TPI* promoter in plasmid pYX212 (R&D systems): pBH2294 was digested with *Aat*II and *Bam*HI and inserted between the corresponding sites in pYX212, resulting in pBH2320. pBH2320 was subsequently digested with *Eco*RI, sticky ends were filled in using Klenow, and following digestion with *Afl*III, a *Hpa*I-*Afl*III fragment from pBH2178 was inserted, yielding pBH2330. HA-tagging of *YGL084c* was carried out by cloning of a *Nco*I-*Cla*I fragment from pBH2310, where the sticky end of the *Nco*I site had been filled in by Klenow treatment, between the *Cla*I site and the *Sma*I site of pBH2330, resulting in pBH2344. This plasmid is thus derived from the 2μ-based vector pYX212 and carries *YGL084c* behind the strong *TPI* promoter. A 2μ-based plasmid carrying the HA-tagged *YGL084c* behind its

own promoter was also generated; pCL577 was made by inserting a 1080 bp *Afl*III-*Sac*I fragment from pBH2344 between the corresponding sites of pBH2178. To generate pCL583, pCL577 was digested with *Kpn*I and *Sac*I and inserted between the corresponding sites in pRS316 (Sikorski and Hieter, 1989). This construct contains *YGL084c*-HA on a centromere-based plasmid.

A chromosomal copy of *YPL189w* was cloned by a combination of PCR and GAP-repair: A 2375 bp fragment, covering from position -2323 to +52, relative to the start codeine, was amplified by PCR, introducing a *Bam*HI site in the downstream end of the fragment. The fragment was digested with *Hind*III and *Bam*HI and inserted between the corresponding sites of pFN8 (Nagawa and Fink, 1985), resulting in plasmid pBH2262. The entire promoter region of *YPL189w* fused to *lacZ* in pBH2262 was sequenced and found to be identical to the already published sequence. A 829 bp *Bam*HI-*Hind*III fragment from pBH2262 was subsequently inserted into the corresponding sites in pRS316 (Sikorski and Hieter, 1989), yielding pCL409. A 600 bp fragment covering from position +1990 to +2589, relative to the start codeine, was amplified by PCR. The fragment was blunt-end-inserted into the *Eco*RV site in pBC sk+ (Stratagene), giving pBH2097. pCL439 was made by insertion of a *Bam*HI-*Hinc*II fragment from pBH2097 into *Eco*ICRI and *Bam*HI-digested pCL409. pCL478, containing the gap-repaired copy of *YPL189w* along with its promoter, was made as follows: pCL439 was digested with *Bam*HI and *Bst*XI and introduced into yeast strain W303-1A, selecting for *URA3*. After recovery from yeast, the plasmid was propagated in *E. coli*. pCL448 was constructed as follows: A 810 bp *Pvu*II-*Bam*HI fragment from pBH2262 was inserted into *Sma*I-*Bgl*II-digested pFA6-KanMX3 (Wach *et al.*, 1994). The resulting plasmid, pCL430, was digested with *Eco*RI and a 527 bp *Eco*RI fragment from pBH2097 was inserted, giving pCL448. A high-copy plasmid containing *YPL189w* was made as follows: A 3.4 kb *Cla*I fragment from pCL478 was inserted into the *Cla*I site of pRS426 (Christianson *et al.*, 1992), giving pCL489. pGut2-2 (Rønnow and Kielland-Brandt, 1993) was digested with *Sna*BI and re-ligated, resulting in pBH2020. pBH2020 was subsequently digested with *Pvu*II and *Bam*HI and inserted between the *Sma*I and *Bam*HI sites of pRS306 (Sikorski and Hieter, 1989) to give pBH2038. A 819 bp *Hind*III-*Spe*I fragment from pGut1-6 (Rønnow, 1992) was inserted between the *Hind*III and *Spe*I sites of pRS306 (Sikorski and Hieter, 1989), and the resulting plasmid was digested with *Spe*I and *Not*I and the latter site was filled in using Klenow. Then a 1453 bp *Pvu*II-*Spe*I fragment from pGut1-6 (Rønnow, 1992) was inserted into the linearised plasmid. This resulted in pCL370.

Kinetics of glycerol uptake

Cells were grown in complex medium and harvested in mid-exponential phase, regardless of the carbon source or the presence of salt stress, by centrifugation, washed twice and re-suspended to a final concentration of ~ 30 mg dry wt. ml^{-1} in ice-cold distilled water. In the case of salt-grown cells, washing, re-suspension and assays were carried out at the same NaCl concentrations. Initial uptake rates of glycerol were determined according to the methodologies described before (Lages and Lucas, 1997), using [^{14}C]glycerol solutions with variable specific activity, ranging from 0.2 to 50 mM with 2800 to 250 dpm/nmol, respectively. Computer regression analysis program GraphPad PRISM^R (GraphPad Software, Inc.) was used to calculate kinetic parameters.

Glycerol accumulation ratios were determined as described by Lages and Lucas (1997). The efflux of radiolabelled glycerol was assayed with 50 μM CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone), a protonophore which was also used for control, as an inhibitor to prevent accumulation.

Glycerol-induced proton uptake was measured as described by Lages and Lucas (1997). Cells were grown in glucose-based complex medium (YPD) supplemented with 1M NaCl and 15 mM glycerol at 30°C. Initial uptake rates of protons were calculated as the change of the tangential slope of the alkalinisation curve, immediately after addition of 10 mM glycerol to the cell suspension, and normalised to cell dry weight. Calibration of pH variations was performed using 10 μl 10.0 mM HCl.

Glycerol kinase assays

Glycerol kinase activity in cell-free extracts was determined according to Krakow and Wang (1990). This method is based on glycerol 3P-dehydrogenase (Boehringer Mannheim 127752)-mediated glycerol 3P oxidation. NAD^+ reduction was followed at 340nm. The reaction mixture was composed of 667 μl buffer (0.3M glycine, 0.7M hydrazine, pH 9.0), 33 μl NAD, 33 μl glycerol (0.3M), 33 μl MgCl_2 (0.15M), 10 to 20 μl cell extract and ultra-pure water to 1 ml volume. ATP (0.15M) and glycerol were alternatively used as starters. Cell-free extracts were prepared by centrifuging and washing twice 250 ml cell culture at 7000 rpm, 2 min at 4°C. Pellets were either used directly or kept frozen at -70°C . Prior to utilisation, these were incubated in buffer (50 mM

MES, 1 mM EDTA, 150 mM (NH₄)₂SO₄, NaOH to pH 6.0 and freshly prepared DTT to 1 mM, broken in a bead mill (1.5 g 0.5 mm Ø glass beads) for 4 × 1 min, intercalated with 1 min rest on ice, followed by centrifugation (1500 g, 15 min, at 4°C). CompleteTM Boehringer Mannheim protease inhibitor (CAT# 1697498) was added. Protein content in extracts was quantified using Folin-Ciocalteu reagent.

Subcellular fractionation

Strain W3124 (*prb1 pep4 prc1*) transformed with pCL583 was grown in a glucose-based complete medium with glutamate as nitrogen source to late exponential phase (OD₆₀₀ ~2), and the cells were broken with glass beads as described (Villalba *et al.*, 1992). Cell debris was removed by 15 min centrifugation at 700 g and total membranes were collected by centrifuging the supernatant for 60 min at 40,000 rpm (Beckman 60 Ti rotor). Sucrose gradient fractionation of membranes and determination of marker enzyme distribution were carried out according to Villalba *et al.* (1992). Gup1p distribution was detected with Western blotting and ECL+Plus chemiluminescence according to the manufacturer (Amersham Pharmacia). The primary antibody was high-affinity rat monoclonal antibody directed against the HA-tag (Boehringer Mannheim) and the secondary antibody was peroxidase-coupled anti-rat IgG (Amersham Pharmacia).

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Table 1. Positions of transposon insertions

Tn- mutant strain	Affected gene	ORF length (amino acids)	Position of transposon insertion ^a	Rescue strain ^b
BHY13	<i>YDL074c</i>	700	1463 T (o.f.)	BHY20-8C
BHY21	<i>YPL180w</i>	799	2326 C (o.f.)	BHY27-5B
BHY22	<i>GUP1</i>	560	165 C (i.f.)	BHY29-2C
BHY23	<i>SPT7</i>	1332	1803 A (i.f.)	BHY25
BHY24	<i>YDL074c</i>	700	1463 T (o.f.)	BHY26-5D

^a Transposon is inserted after indicated nucleotide. o.f. indicates an out-of-frame fusion. i.f. indicates an in-frame fusion. ^b *LEU2 ura3* derivatives of the original transposon mutants, see Experimental procedures. A *URA3*-based plasmid was introduced into each rescue strain allowing rescue of part of the disrupted gene for identification.

Table 2. Kinetic parameters of initial [^{14}C]glycerol uptake at 30°C and pH 5.0 in cells grown in ethanol-based complex medium (YP-ethanol)

Relevant Genotypes	K_m (mM)	V_{\max} ($\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g dw}^{-1}$)	D ($\text{l} \cdot \text{h}^{-1} \cdot \text{g dw}^{-1}$)
Wild type	1.1 ± 0.2 (3)	277 ± 26 (3)	0.010 (1)
<i>gup1</i>	1.5 ± 0.5 (4)	181 ± 12 (4)	0.009 (1)
<i>gup1 fps1</i>	1.4 ± 0.6 (3)	137 ± 10 (3)	0.008 (1)
<i>gup1 ypl189w</i>	1.6 ± 0.7 (3)	169 ± 17 (3)	0.008 (1)
<i>gut1</i>	1.3 ± 0.4 (4)	205 ± 17 (4)	0.009 (4)
<i>gut1 gpd1</i>	1.8 ± 0.6 (4)	248 ± 36 (4)	0.011 (3)
<i>gut1 gpd1 gup1</i>	- (3)	- (3)	0.007 (1)
<i>gut1 gup1 ypl189w</i>	- (2)	- (2)	0.007 (1)

All strains are derived from W303-1A; only relevant genotypes are shown.

- means that no active uptake was detected.

Numbers in parenthesis indicate numbers of independent assays

Table 3. Kinetic parameters of initial uptake rates of [^{14}C]glycerol at 30°C and pH 5.0 in cells grown in ethanol-based complex medium (YP-ethanol)

Relevant genotypes	K_m (mM)	V_{\max} ($\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g dw}^{-1}$)	D ($\text{l} \cdot \text{h}^{-1} \cdot \text{g dw}^{-1}$)
Wild type	1.1 ± 0.2 (3)	277 ± 26 (3)	0.010 ± 0.001 (3)
<i>gut1</i>	1.3 ± 0.4 (4)	205 ± 17 (4)	0.009 ± 0.001 (4)
<i>gut2</i>	1.5 ± 0.7 (3)	212 ± 48 (3)	0.008 ± 0.002 (3)
<i>gpp1</i>	1.6 ± 0.7 (3)	326 ± 62 (3)	0.011 ± 0.002 (3)
<i>gpp2</i>	1.9 ± 0.2 (2)	318 ± 20 (2)	0.009 ± 0.001 (2)
<i>gpp1 gpp2</i>	1.5 ± 0.3 (3)	250 ± 27 (3)	0.007 ± 0.001 (3)
<i>gpd1</i>	1.3 ± 0.3 (2)	256 ± 27 (2)	0.008 ± 0.001 (2)
<i>gpd2</i>	1.2 (1)	252 (1)	0.009 (1)
<i>gpd1 gpd2</i>	1.6 ± 0.4 (3)	237 ± 30 (3)	0.013 ± 0.001 (3)

All strains are derived from W303-1A; only relevant genotypes are shown.

Numbers in parenthesis indicate numbers of independent assays

Table 4. Estimation of the V_{\max} ($\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g dw}^{-1}$) of glycerol uptake into salt-stressed or unstressed cells grown in glucose-based complex medium at 30°C and pH 5.0 by measuring uptake of [^{14}C]glycerol at an extracellular concentration of 2mM

Relevant Genotypes	Growth medium (YPD)	
	Glucose	Glucose
	+ 1 M NaCl + 15 mM Glycerol	
Wild type	<50	<50
<i>gpd1 gpd2</i>	<50	630 ± 18 (3)
<i>gup1</i>	<50	<50
<i>gut1</i>	<50	<50
<i>gup1 gut1</i>	<50	<50
<i>gpd1 gup1</i>	<50	182 ± 0.5 (2)
<i>gup1 ypl189w</i>	<50	<50
<i>gpd1 gup1 ypl189w</i>	<50	<50
<i>gpd1 gpd2 gup1</i>	<50	379 ± 37 (2)

All strains are derived from W303-1A; only relevant genotypes are shown.

Numbers in parenthesis indicate numbers of independent assays

Table 5. Strains used in this study

W303-1A	<i>MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100</i>	Thomas and Rothstein, 1989
W303-1B	Isogenic to W303-1A but <i>MAT?</i>	Thomas and Rothstein, 1989
W303-2n	Diploid of W303-1A and W303-1B	Thomas and Rothstein, 1989
UTL-7A	<i>MATa ura3-52 leu2 trp1 gal2 gal10</i>	Heinisch, J.
YSH481	Isogenic to W303-1A but <i>MATα gpd1::LEU2</i>	Hohmann, S.
YSH6.142-3D	Isogenic to W303-1A but <i>MATα gpd1::TRP1 gpd2::URA3</i>	Hohmann, S.
YSH392	Isogenic to W303-1A but <i>gpd1::TRP1</i>	Hohmann, S.
YSH6.141-4A	Isogenic to W303-1A but <i>gpd2::URA3</i>	Hohmann, S.
YSH642	Isogenic to W303-1A but <i>gpd1::TRP1 gpd2::URA3</i>	Hohmann, S.
	Isogenic to W303-1A but <i>MATα gpp1::KanMX4</i>	Påhlman, A.-K. and Adler, L.
	Isogenic to W303-1A but <i>gpp2::KanMX4</i>	Påhlman, A.-K. and Adler, L.
	Isogenic to W303-1A but <i>gpp1::KanMX4 gpp2::HIS3MX6</i>	Påhlman, A.-K. and Adler, L.
W3124	<i>MATa ura3-52 leu2-3,112 his3-200 prc1Δ::HIS3 prb1Δ::LEU2 pep4-1137</i>	Winther, J.R.
BHY13	Isogenic to W303-1A but <i>MATα gpd1::TRP1 gpd2::URA3 ydl074c::Tn-LEU2</i>	This study
BHY20-8C	<i>MATα ura3 leu2 trp1 ade2 gpd1::TRP1 ydl074c::Tn-LEU2</i>	This study
BHY21	Isogenic to W303-1A but <i>MATα gpd1::TRP1 gpd2::URA3 ypl180w::Tn-LEU2</i>	This study
BHY22	Isogenic to W303-1A but <i>MATα gpd1::TRP1 gpd2::URA3 ygl084c::Tn-LEU2</i>	This study
BHY23	Isogenic to W303-1A but <i>MATα gpd1::TRP1 gpd2::URA3 spt7::Tn-LEU2</i>	This study
BHY24	Isogenic to W303-1A but <i>MATα gpd1::TRP1 gpd2::URA3 ydl074c::Tn-LEU2</i>	This study
BHY26-5D	<i>MATa ura3 leu2 trp1 his3 ade2 gpd1::TRP1 ydl074c::Tn-LEU2</i>	This study
BHY27-5B	<i>MATα ura3 leu2 trp1 his3 ade2 gpd1::TRP1 ypl180w::Tn-LEU2</i>	This study
BHY29-2C	<i>MATa ura3 leu2 trp1 his3 gpd1::TRP1 ygl084c::Tn-LEU2</i>	This study
BHY28-8C	<i>MATa ura3 leu2 trp1 his3 gpd1::TRP1 spt7::Tn-LEU2</i>	This study
BHY39	Isogenic to W303-1A <i>GUT2</i>	This study
BHY40	Isogenic to W303-1A but <i>gut2</i>	This study
BHY51	Isogenic to W303-1A but <i>MATα gpd1::LEU2 gut1::KanMX</i>	This study
BHY52	Isogenic to W303-1A but <i>fps1::HIS3</i>	This study
BHY54	Isogenic to W303-1A but <i>ygl084c::His5⁺</i>	This study
BHY56	Isogenic to W303-1A but <i>MATα gpd1::LEU2 ygl084c::His5⁺</i>	This study
BHY58	Isogenic to W303-1A but <i>MATα ygl084c::His5⁺</i>	This study
BHY61-1A	<i>MATα ura3 leu2 trp1 his3 ade2 ygl084c::his5⁺ fps1::HIS3</i>	This study

BHY65-1C	<i>MATa ura3 leu2 trp1 his3 ade2 gut1 ygl084c::his5⁺</i>	This study
BHY66	Isogenic to W303-1A but <i>MATα gpd1::LEU2 gut1::KanMX ygl084c::His5⁺</i>	This study
BHY67	<i>MATa ura3 leu2 trp1 his3 ade2 gut1 ygl084c::his5⁺ ypl189w::KanMX</i>	This study
CLy1	Isogenic to W303-1A but <i>gut1</i>	This study
CLy2	Isogenic to W303-1A <i>GUT1</i>	This study
CLy3	Isogenic to W303-1A but <i>ygl084c::His5⁺ ypl189w::KanMX</i>	This study
CLy4	Isogenic to W303-1A but <i>gpd1::LEU2 ygl084c::His5⁺ ypl189w::KanMX</i>	This study
CLy5	Isogenic to W303-1A but <i>ypl189w::KanMX</i>	This study

Figure legends

Fig. 1. Phenotypes of mutants isolated in a *gpd1 gpd2* strain. Cells were grown in YPD at 30°C to OD₆₀₀ ~ 3 and diluted to OD₆₀₀ = 1. Ten µl of a 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ dilution were spotted on different media. Upper panel: Growth on glycerol as carbon and energy source. Lower panel: YPD plates containing 1 M NaCl and supplemented with 10 mM glycerol. Lane 1, parental strain (YSH6.142-3D); Lane 2, BHY13; Lane 3, BHY21; Lane 4, BHY22; Lane 5, BHY23; Lane 6, BHY24. Plates were incubated at 30°C for 72 h and 48 h, respectively.

Fig. 2. Alignment of Gup1p with the close homologue Ypl189wp (Gup2p) and Spac24h6-01cp from *S. pombe* using LASERGENE from DNA STAR, Inc., Madison. **A**, Amino acids that are conserved between at least two of the sequences are shown in closed boxes. **B**, Hydrophobicity profiles calculated according to Kyte and Doolittle (1982).

Fig. 3. Plasmid complementation of strain BHY29-2C, disrupted in *YGL084c* (*GUPI*) by the transposon. **A**, Cells were grown and spotted as in described in the legend to Figure 1. Upper panel: Growth on glycerol as carbon and energy source. Lower panel: YPD containing 1 M NaCl and supplemented with 10 mM glycerol. Lane 1, W303-1A (wt); Lane 2, BHY29-2C transformed with pRS316 (vector without insert). Lane 3, BHY29-2C transformed with pBH2178 (low-copy plasmid with *GUPI*). Lane 4, BHY29-2C transformed with pBH2198 (high-copy plasmid with *GUPI*). **B**, BHY29-2C was grown at 30°C to OD₆₀₀ ~3 and diluted to OD₆₀₀ = 0.01. A volume of 1 ml was spread on a YPD plate containing 1 M NaCl. A filter disk containing 20 µl of 87% glycerol was placed in the middle of the plate. Panel 1, BHY29-2C transformed with pRS316 (vector without insert); Panel 2, BHY29-2C transformed with pBH2178 (low-copy *GUPI*); Panel 3, BHY29-2C transformed with pBH2198 (high-copy *GUPI*). Plates were incubated at 30°C for 48 hr.

Fig. 4. Complementation of the *gup1* mutant strain with *GUPI*. Cells were grown and spotted as described in the legend to Figure 1. Upper panel: Growth on glycerol as carbon and energy source. Lower panel: YPD containing 1 M NaCl and supplemented with 10 mM glycerol. Lane 1, W303-1A (wt). Lane 2, BHY54 (*gup1*Δ) transformed with pRS316 (vector without insert). Lane 3, BHY54 transformed with pBH2178 (low-copy plasmid with *GUPI*). Lane 4, BHY54 transformed with pBH2198 (high-copy plasmid with *GUPI*).

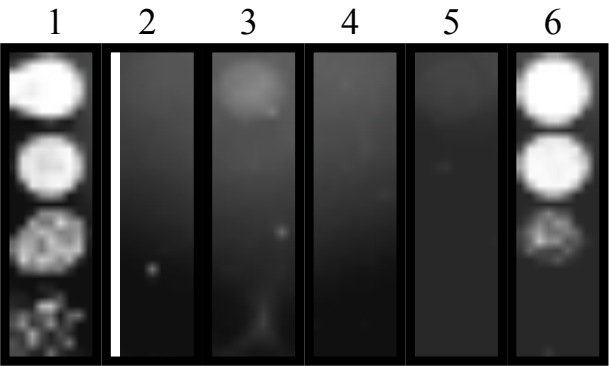
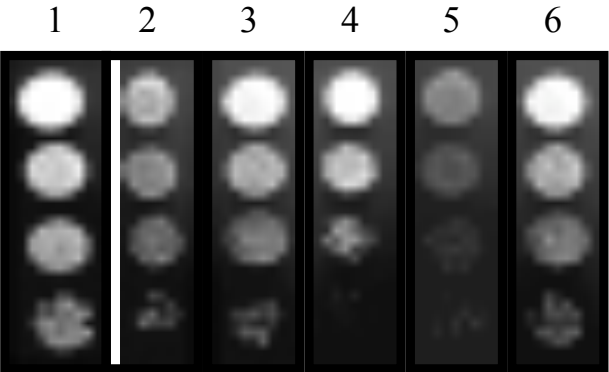
Fig. 5. Eadie-Hofstee plots of initial uptake rates of [14 C]glycerol at pH 5.0 and 30°C by W303-1A (wt), CLy1 (*gut1*Δ), BHY54 (*gup1*Δ), BHY51 (*gpd1*Δ *gut1*Δ) and BHY66 (*gpd1*Δ *gut1*Δ *gup1*Δ) harvested during exponential growth on 2% ethanol.

Fig. 6. Radio-labelled glycerol accumulation ratios at pH 5.0 and 30°C in W303-1A (wt), BHY54 (*gup1*Δ), and CLy1 (*gut1*Δ), harvested during exponential growth on 2% ethanol (•). Addition of 50 μM CCCP as indicated by an → (○). Incubation in the presence of 50 μM CCCP (Δ).

Fig. 7. Subcellular localisation of Gup1p as investigated by sucrose gradient centrifugation. Total membranes from a protease-deficient yeast strain (W3124) transformed with a Gup1p-HA-expressing plasmid were isolated and separated on a sucrose gradient. Fractions were collected and analysed for the activity of (open circles) plasma membrane H⁺-ATPase activity (A), endoplasmic reticulum NADPH-cytochrome oxidase (B), mitochondrial cytochrome oxidase (C) and vacuolar α-mannosidase (D). The distribution of Gup1p was determined by Western blotting and quantification of the bands (closed circles). The triangular symbols in panel D represent the total protein concentration in mg/ml.

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A

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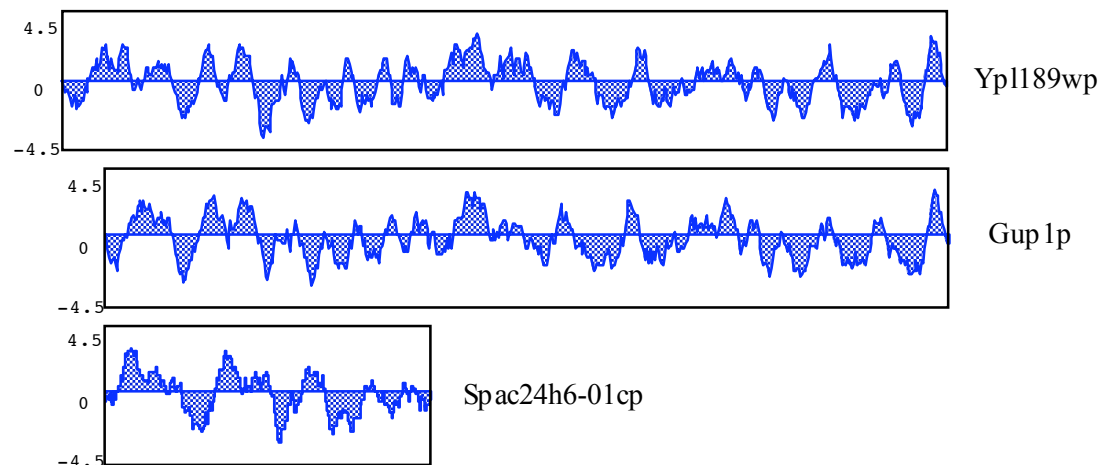
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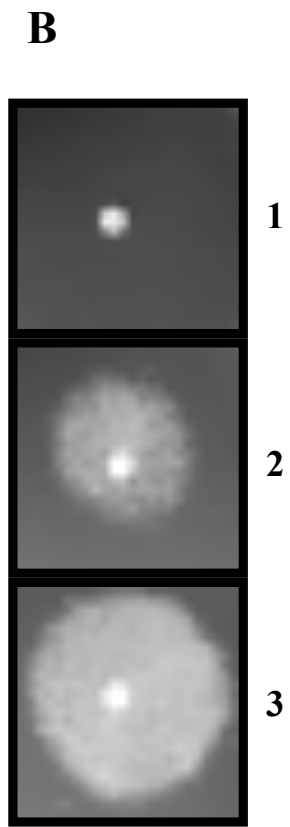
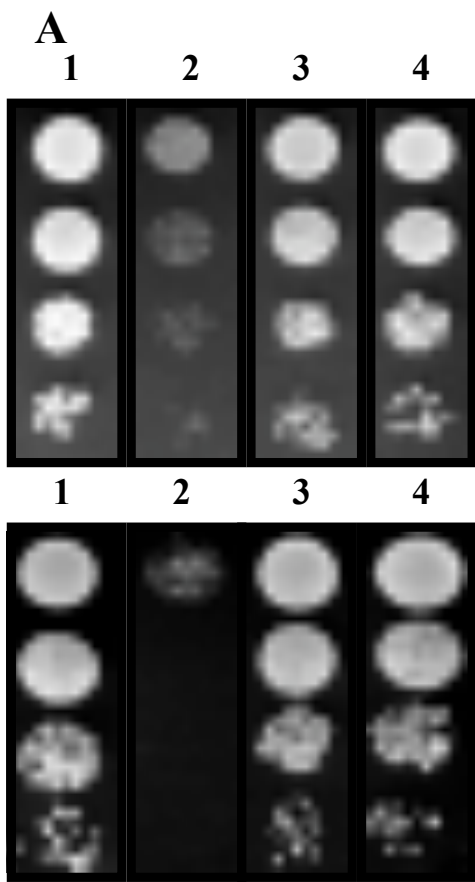
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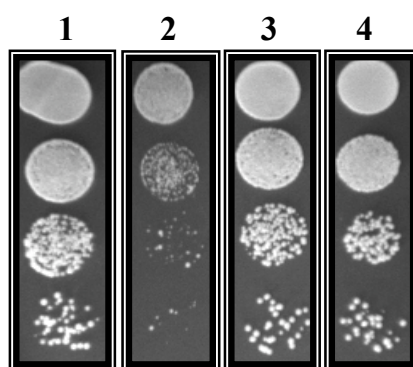
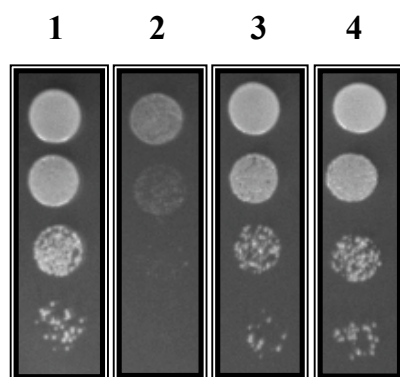
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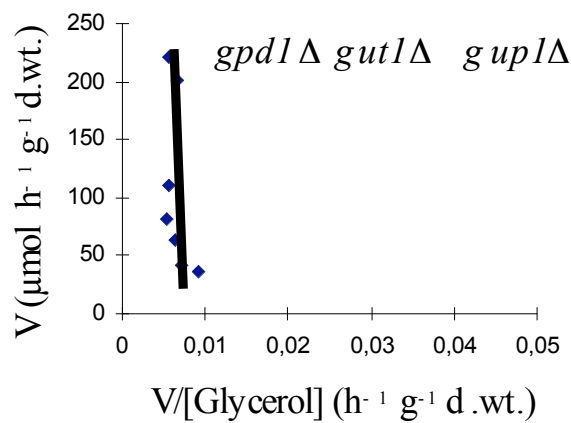
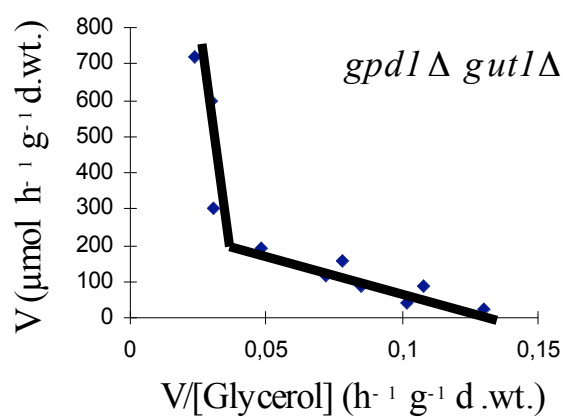
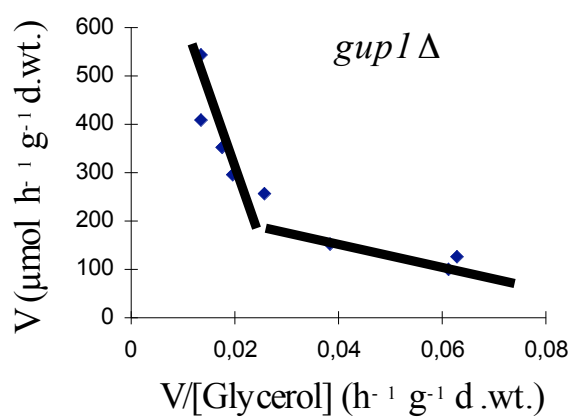
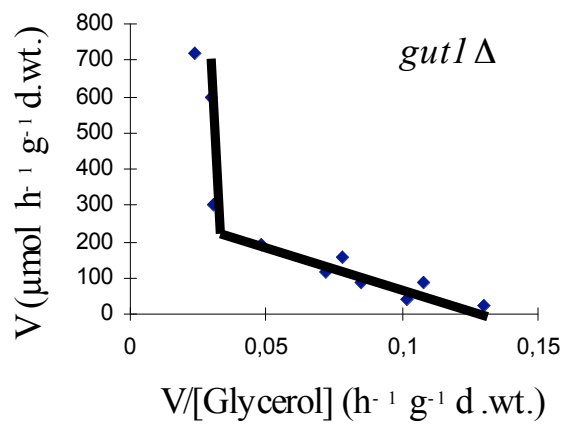
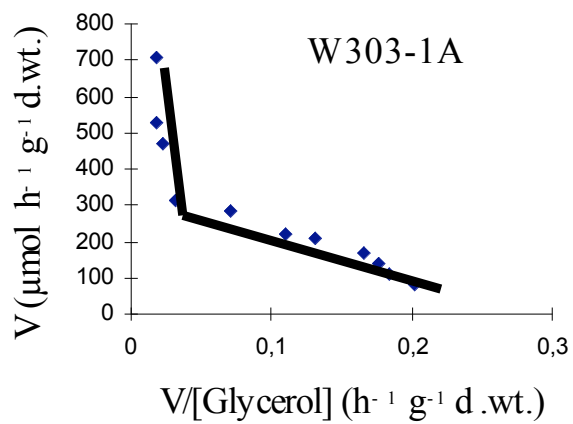
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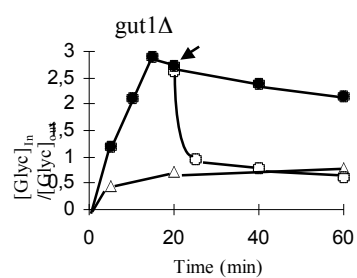
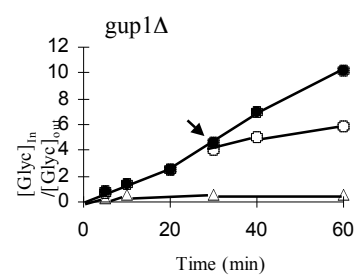
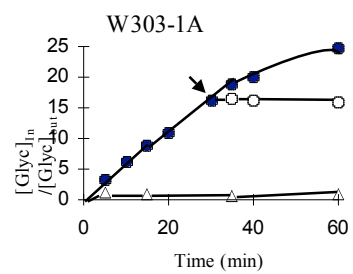
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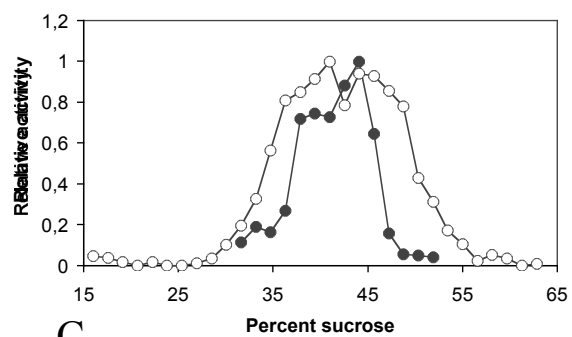




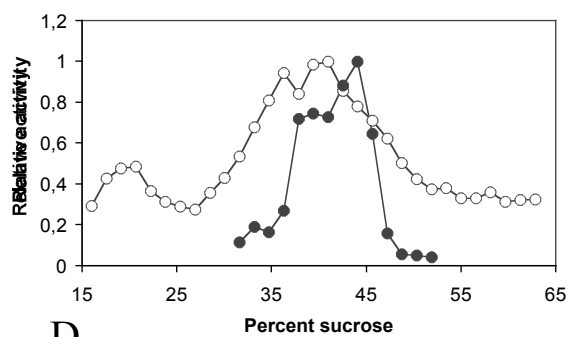




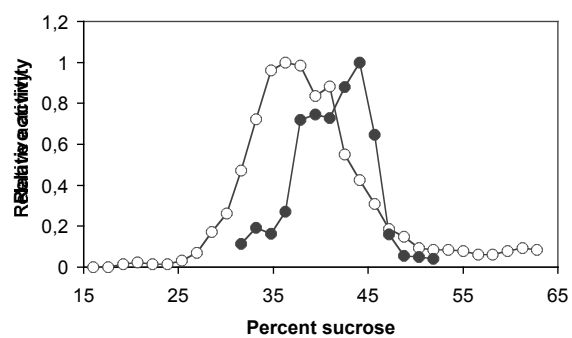
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B



C



D

